Association between neonatal resuscitation and a single nucleotide polymorphism rs1835740

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Short Title: Neonatal resuscitation is associated with SNP rs1835740

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Authors' contributions

Dr Odd had primary responsibility for the preliminary data analysis and writing the manuscript. Drs Váradi, Rajatileka, Molnár and Luyt participated in the development of the protocol and analytical framework for the study and contributed to the writing of the manuscript.

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ABSTRACT

Aim: The aim of this work is to test if three single nucleotide polymorphisms (SNPs) implicated in glutamate homeostasis or signalling and cellular survival are associated with birth condition.

Methods: This study is drawn from the Avon Longitudinal Study of Parents and Children. 7611 term infants were genotyped and patient outcome data retrieved from routine medical records. Exposure measures were the presence of one or more minor alleles in one of 3 SNPs (rs2284411, rs2498804, rs1835740). The primary outcome was the need for resuscitation at birth.

Results: For SNP rs1835740, infants homozygous for the minor allele compared to wild-type were more likely to need resuscitation (9.2% vs. 7.0%, p=0.041) while the odds ratio for resuscitation was associated with each increasing minor allele (OR 1.17 (1.01 to 1.35)). Population attributable risk fraction was 6.5%. There was no evidence that the other two SNPs investigated were associated with birth condition.

Conclusions: We have tested three candidate SNPs to measure any association with birth condition. The study revealed that the rs1835740 was associated with the need for resuscitation and Apgar scores, with a substantial population impact.

Key Words: Asphyxia Neonatorum, Brain, Cohort Studies, Hypoxia-Ischemia, Glutamate, Polymorphism

KEY NOTES

- We have tested three candidate SNPs to measure any association with birth condition.
- There was evidence that one (rs1835740) was associated with the need for resuscitation and Apgar scores, with a substantial population impact.
- This is some of the first *in vivo* evidence implicating glutamate in perinatal asphyxia.

INTRODUCTION

Most infants tolerate the birth process well, but ~7% require some degree of resuscitation and ~0.5% of all births go on to develop immediate signs of brain damage.(1) We(1) and others(3) have been attempting to quantify the effect and wider impact of physiological compromise during the birth process. It is well recognized that infants born in poor condition, and in particular those who go on to develop signs of neurological impairment shortly after birth, have increased risk of movement disorders (cerebral palsy) and intellectual disability.(4) The outcomes is also devastating for the patients' family and society.(5,6) We have shown that the neurological impairment persist through childhood(1) into adulthood.(2) Our work has also suggested important effects on social measures of outcome such as income, education and marriage.(7) Importantly, we have reported long-term impacts in infants who only required brief life support at birth and had no noticeable problems in the neonatal period,(1,7) consistent with the hypothesis of a "Continuum of Reproductive Casualty."(8)

Hypoxic events are frequently associated with birth complications.(9) It has been proposed that the major excitatory neurotransmitter glutamate and its receptors play a key role in the neural damage that occurs during hypoxic episodes in the developing brain(10). Indeed, glutamate concentration has been found to be markedly increased in the cerebrospinal fluid(11) and also in the basal ganglia of asphyxiated newborn infants measured *in vivo* by Magnetic Resonance Spectroscopy.(12) While *in vivo* evidence is limited, single nucleotide polymorphisms (SNPs) affecting gene transciption and protein function in glutamatergic signalling and cell survival pathways may play a key role in cerebral recovery after hypoxic events.(13)

In acute ischaemic brain injury in newborns, the primary step leading to excitotoxicity and neuronal death is the excessive activation of glutamate receptors, principally the synaptic and GluN2B subunit-containing extrasynaptic *N*-methyl-D-asparate receptors (NMDARs).(13,14) GluN2B is highly expressed during the perinatal developmental period and deletions in the GluN2B gene are

associated with cognitive disability in children.(15) Intriguingly, the functional polymorphism rs2284411 in GluN2R has an impact on susceptibility to neurodevelopmental disorders in children.(16)

The serine/threonine kinase Akt (or protein kinase B, PKB) mediates neuronal cell survival in response to growth factors in the developing central nervous system(17) and involved in group I metabotropic glutamate receptor-linked signalling.(18) Akt has been reported to enhance cell survival in neurones by blocking induction of apoptosis.(19) The SNP rs2498804 in the Akt gene have been found to impair Akt function resulting in increased apoptosis.(21) In patients with schizophrenia who were born after obstetric complications this variant was more common, suggesting that this SNP may increase foetal vulnerability to hypoxia.(20)

SNP rs1835740, is located between astrocyte elevated gene 1 (*AEG1*)(21) and plasma glutamate carboxypeptidase (*PGCP*).(22) AEG1 down regulates the major glutamate transporter, the excitatory amino-acid transporter-2 (EAAT2), in the central nervous system (CNS) and PGCP is involved in glutamate synthesis. rs1835740 was demonstrated to be a *cis*-acting regulator of *AEG-1* and the risk A allele (in forward orientation T allele) was associated with higher expression levels of AEG1 and the development of migraine(22) and cluster headaches, suggesting a link between the rs1835740 variant and impaired regulation of glutamate levels in the CNS.(20,21)

We hypothesise that these functional SNPs affecting glutamatergic signalling, homeostasis and cell survival in the CNS may influence infants' susceptibility to the birth process and subsequent development of neurological impairment. Identification of a SNP related to birth condition could provide both insight into presumed pathophysiological pathways, as well as a marker to assess the true impact of perinatal asphyxia at a population level.

PATIENTS AND METHODS

Cohort selection

This study is drawn from the Avon Longitudinal Study of Parents and Children (ALSPAC), an on-going study containing data on over 14,000 infants(23) or which there were 7611 term (36^{+0} to 42^{+6} weeks gestation) infants who were genotyped using the Illumina HumanHap550 quad genome-wide SNP genotyping platform (Illumina UK, Little Chesterford, UK).(24) Further information about the study can be found on the ALSPAC website which contains details of all the data that is available through a fully searchable data dictionary [http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary/]. Within the SNPs measured by the ALSPAC cohort were 3 potential functional SNPs directly measured in these pathways of interest.

Data on cohort members have been retrieved from routine medical records. Ethical approval for the study was obtained from the ALSPAC Law and Ethics Committee and the Local Research Ethics Committees.

Gene Data

Exposure measures were the presence of one or more minor alleles in one of three SNPs (rs2284411, rs2498804, rs1835740; Table 1) proposed to modify the association between hypoxic stress and neuronal injury.

Outcome measures

The primary outcome, consistent with our previous work in poor condition at birth,(1) was (i) the need for resuscitation at birth. This was defined as the need for positive pressure respiratory support using a face mask or endotrachael tube; and/or cardiac compressions at birth. Two secondary measures of birth condition, also consistent with our previous work(1) were also included (ii) Duration of poor birth condition defined as the time to reach an Apgar score of 7-10 (a normal score)(25) (taken from Apgars documented at 1, 5 and 10 minutes).(1,2,7) (iii) Hypoxic-Ischaemic Encephalopathy (HIE). HIE may be defined as “a subnormal level of consciousness or seizures, and often accompanied by difficulty with initiating and maintaining respiration and depression of tone and reflexes”.(26) In this work, infants were diagnosed if the infant had seizures, jitteriness, high pitched cry, hypo- or hypertonia or hyper-reflexia during the neonatal period following the need for resuscitation).(1) The demographics of the infants included in the

study are described in Table 2.

Statistical Methods

To assess the association between the individual SNP genotypes and the three outcomes, univariable comparisons were performed for each genotype. Logistic or ordinal regression models (as appropriate) were then derived to assess the association of each increasing minor-allele with the three outcomes. Population attributable risks were calculated from the final logistic regression model.

Four sensitivity analyses were performed to investigate alternative mechanisms for any SNP association (e.g. effects on gestational age at birth). In the first the association between the three SNPs and resuscitation was adjusted for antenatal (gender, parity, maternal hypertension) and then intrapartum factors (gestational age, birth weight, length and head circumference, mode of birth and neonatal sepsis). Adjustment for possible confounders was performed by adding the variables described above to the models, in blocks of common variables (e.g. antenatal). Ordinal variables were tested for linearity and included in the model as linear terms if appropriate. In the second sensitivity analyses we investigated if the association between the SNPs and the risk of resuscitation was modified if the infant was at higher of risk of poor condition at birth (defined as: breech deliveries, emergency caesarean sections, instrumental deliveries, low birth weight (<10th centile for gestational age), maternal hypertension).(27) In the third analysis we repeated the analysis including all SNPs in a single model, while in the fourth we investigated if any association was modified by the infants' gender.

All analyses were conducted with Stata 10 software (Stata Corp, TX, USA). All data are presented as odds ratio (OR) (95% confidence interval (CI)), mean (SD), mean difference (95% CI), median (interquartile range (IQR)), or number (percent).

RESULTS

There was no evidence that the SNPs measured were not in Hardy-Weinberg equilibrium, nor was

there any evidence that any of the SNPs were in linkage disequilibrium with each other (all comparisons; p>0.10). In the univariable analysis, there was no evidence that rs2284411 or rs2498804 were associated with need for resuscitation, time to a normal Apgar score or development of hypoxic-ischemic encephalopathy (Table 3). However, for rs1835740 the need for resuscitation (p=0.041) and the time to achieve a normal Apgar score (p=0.020) differed by genotype (Table 3). Infants homozygous for the minor allele compared to wild-type were more likely to need resuscitation (9.2% vs. 7.0%) and took longer to achieve an Apgar score of 7-10 (number with low Apgar score beyond 5 minutes; 1.2% vs. 0.7%). There was no clear evidence that rs1835740 was associated with the risk of hypoxic-ischaemic encephalopathy.

In the logistic regression neither rs2284411or rs2498804 were found to be associated with the need for resuscitation, time to a normal Apgar score or hypoxic-ischaemic encephalopathy (Table 4). However, consistent with the univariable analysis, each increasing minor allele of rs1835740 was associated with increased risk of resuscitation (OR 1.17 (1.01 to 1.35)) and time to achieve a normal Apgar score (OR 1.17 (1.03-1.34)), but not with hypoxic-ischaemic encephalopathy (OR 0.92 (0.49 to 1.75)). A model containing all three SNPs produced near-identical results (e.g. rs1835740; OR 1.17 (1.01 to 1.35)). Population attributable risk fractions (PARF) were 3.0% for rs2284411, 3.1% for rs2498804 and 6.5% for rs1835740.

In the sensitivity analysis, adjusting for antenatal or intrapartum factors did not substantially modify any relationship seen in the initial model, with no association between 2 of the SNPs (rs2284411, rs2498804) and the need for resuscitation (Table 5). The association between rs1835740 and resuscitation remained in both the model correcting for antenatal factors (OR 1.17 (1.01-1.35)) and antenatal/intrapartum factors (OR 1.18 (1.00-1.38)). There was no evidence that high risk delivery modified the relationship between any of these SNPs and the need for resuscitation (rs2284411, p=0.657; rs2498804, p=0.958; rs1835740, p=0.401). Using a model with all 3 SNPs in showed no substantial difference from the main analysis, with only evidence that rs1835740 was associated with the need for resuscitation (OR 1.17 (1.01 to 1.35)), while there was no evidence that gender modified any association between the SNPs and the need for resuscitation (all p values greater

than 0.5).

DISCUSSION

In this cohort one of the SNPs, rs1835740, implicated in glutamate homeostasis and signalling showed an association with birth condition in both the need for resuscitation and the length of time the infant took to achieve a normal Apgar score (Table 3). This is, to our knowledge, the first time a functional SNP in the excitatory neurotransmitter pathway has been associated with this important perinatal outcome.

Similarly to the majority of SNPs that have been found to be associated with human complex diseases and traits in genome-wide association studies, rs1835740 is located between two genes (AEG1 and PGCP). AEG1 and PGCP are involved in glutamate homeostasis (22). Genome-wide expression quantitative trait locus (eQTL) analysis in lymphoblastoid cells has indicated that s1835740 is a cis-acting regulator of the former gene (22) and the risk allele A is associated with higher expression levels of AEG-1. Furthermore, it has been shown that AEG-1 downregulates the expression of the glutamate transporter EAAT2 in cultured astrocytes (30). Taking all these together, in infants with the A allele the expression level of AEG-1 is likely to be elevated which in turn can downregulate EAAT-2 resulting in pathological extracellular glutamate levels and increased susceptibility to hypoxia-ischaemiainduced excitotoxicity.(22)

While the underlying pathological mechanisms are not fully understood, it is plausible that the rs1835740 SNP modifies the impact of otherwise physiological levels of hypoxia to produce cerebral glutamate concentrations substantial enough to produce clinical depression at birth. Alternatively, it may modify the infants' reaction to a similar hypoxic insult, resulting in central respiratory depression and corresponding lower Apgar scores, which then require the infant to receive resuscitation. In addition the exact mechanisms of poor birth condition are complex and the causal pathways unclear (e.g. meconium stained liquor is likely to represent the outcome of asphyxia as much as the cause). It is important to note that the population impact of this SNP appears substantial. The population attributable risk fraction estimates the proportion of infants

born in poor condition due to these genes, and in this work, a fraction of over 6%, suggests that this SNP is the cause of 1:16 infants born in poor condition. The presence of Hardy-Weinberg equilibrium in this work suggests the population was not selected prior to birth, or through the methodology. We have previously shown that infants who require resuscitation in this cohort go on to have lower cognitive scores and school performance(1) as well as increased signs of psychiatric disease(4) and so, if causal, this SNP may represent an important cause of childhood morbidity although we do not have the power to test later impacts within this study. The strength of our study is the use of a population-based sample, with prospectively collected data and robust information on potential confounders. The main limitation is the restriction of the genotyping to infants classified to be within the ethnicities covered by the HapMap project.(28) While the three SNPs we have investigated were all measured directly on the infants we were not able to get data on those infants who fell outside the standardising procedure used in the genotyping and so were not able to investigate the effect of ethnicity due to the low levels of variance in the population. Given the lack of dependencies between the SNPs, adjustment for multiple testing in this a-priori work would be over-conservative. Bonferroni correction would propose that a 'significant' p-values cut-off of <0.017 could be used (instead of 0.05). None of our analyses obtained this level of evidence, and this emphases that the association seen in this work may simply be that of chance (false positive error) and further work to confirm or refute this is needed. We need to acknowledge that we have not adjusted the p-values for multiple testing, as all three SNPs were defined a-priori and are not in linkage disequilibrium with each other. Two of the three SNPs (rs2284411 and rs2498804) did not show significant association with birth condition, and thus it is likely that they play no role in the physiological cascade around birth, or that any functional effect they might have is not measurable in this work. However the confidence intervals (for resuscitation and Apgar scores at least) were relatively narrow suggesting we are likely to be missing only a small effect of these two other SNPs, should one exist. However due to the imprecision in the point estimates it is important not to see this work as evidence of no association. Of note, results were identical when the analysis was repeated excluding infants born at 36 weeks.

This work shows that genetic variation has measurable and potentially important impact on perinatal health and the birth process itself, which in turn is associated with poorer cognitive skills, psychiatric morbidity and worse pragmatic, social outcomes.(1,2,7) We also know that interventions around the birth process can modify this risk, with recent work suggesting risk profiling may be of use.(29) Of interest, none of the three SNPs investigated here were associated with a higher risk of an emergency LSCS (all p-values >0.5). Non-invasive fetal genotyping is also becoming possible with recent technical advances giving the possibility of incorporating important SNPs into a risk prediction strategy to reduce perinatal asphyxia by targeted obstetric intervention. We have tested 3 candidate SNPs to measure any association with birth condition, and while there was no significant association between the SNPs in the GluN2B and Akt1 genes with birth condition there was evidence that the rs1835740 was associated with the need for resuscitation and Apgar scores, with an imporant population impact. This association was not confounded by antenatal or intrapartum factors in a sensitivity analysis.

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ABBREVIATIONS

CI - Confidence Interval, ICD - International Classification of Disease, SNP - Single Nucleotide Polymorphism

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Table 1. SNPs included in the analysis

SNP	Location	Proposed function
rs2284411 (g.13866172C>T)	Chromosome 12p13.1: Glutamate receptor, *N*-methyl D-asparate receptor subunit 2B (GluN2B) gene	Affects NMDA receptor function by regulatory effects of GluN2B gene expression(17).
rs2498804 (g.105233095C>A)	Chromosome 14q32.32: V-akt murine thymoma viral oncogene homolog 1 (Akt1) gene	Impairs Akt function, resulting in increased apoptosis in cancer(29) and in multiple sclerosis(40).
rs1835740 (g.98166913T>C)	Chromosome 8q22.1	Down regulates glutamate transport (EAAT2) and promotes excitotoxicity(21).

Table 2. Characteristics of study population (n=7,611)

Measure	Number (%), Mean (95% CI) or Median (IQR)
Antenatal factors	
Gender	
Male	3,896 (51.2%)
Female	3,715 (48.8%)
Primiparous	4,080 (56.0%)
Maternal Hypertension	212 (3.0%)
Non-White Ethnicity	22 (0.3%)
Infants and post-partum factors	
Gestational age	39.7 (1.4)
Birth weight	3472 (484)
Birth length	51.0 (2.4)
Birth head circumference	34.9 (1.4)
Mode of birth	
Spontaneous cephalic	5,374 (76.1%)
Emergency caesarean section	403 (5.7%)
Elective caesarean section	290 (4.1%)
Instrumental	902 (12.8%)
Breech	95 (1.3%)
Neonatal Sepsis	68 (0.9%)
Birth condition	
Resuscitated	529 (7.5%)
Apgar score at 1 min	9 (8-9)
Apgar score at 5 min	10 (9-10)

Numbers are n(%), median (IQR) or mean (SD) as appropriate

Table 3. Associations between the three candidate SNPs and measures of birth condition

SNP	Outcome Measure	n	Homozygous (Wild type)	Heterozygous	Homozygous (Minor Allele)	p_{trend}
rs2284411 (C>T)			n=3,147	n=3,504	n=960	
	Resuscitation	7058	228 (7.8%)	244 (7.5%)	57 (6.4%)	0.19
	Time to Apgar score >6	7053				
	<1 minute		2632 (90.3%)	2945 (90.9%)	821 (91.6%)	0.20
	1-5 minutes		267 (9.2%)	271 (8.4%)	70 (7.8%)	
	>5 minutes		17 (0.6%)	25 (0.8%)	5 (0.6%)	
	Hypoxic-ischaemic Encephalopathy	7611	12 (0.4%)	13 (0.4%)	4 (0.4%)	0.93
rs2498804 (C>A)			n=3,726	n=3,182	n=703	
	Resuscitation	7058	250 (7.3%)	222 (7.5%)	57 (8.7%)	0.27
	Time to Apgar score >6	7053				
	<1 minute		3116 (90.5%)	2690 (91.0%)	592 (90.4%)	0.78
	1-5 minutes		308 (9.0%)	245 (8.3%)	55 (8.4%)	
	>5 minutes		19 (0.6%)	20 (0.7%)	8 (1.2%)	
	Hypoxic-ischaemic Encephalopathy	7611	13 (0.4%)	14 (0.4%)	3 (0.4%)	0.67
rs1835740 (A>G)			n=4,615	n=2,642	n=354	
	Resuscitation	7058	301 (7.0%)	198 (8.1%)	30 (9.2%)	0.04
	Time to Apgar score >6	7053				
	<1 minute		3917 (91.3%)	2193 (90.1%)	288 (88.1%)	0.02
	1-5 minutes		345 (8.0%)	228 (9.4%)	35 (10.7%)	
	>5 minutes		29 (0.7%)	14 (0.6%)	4 (1.2%)	
	Hypoxic-ischaemic Encephalopathy	7611	18 (0.4%)	10 (0.4%)	1 (0.3%)	0.81

Values are number (%)

Table 4. Logistic regression models for the association between the three candidate SNPs and measures of birth condition, for each increasing allele.

SNP	Resuscitation OR (95% CI)	Time to Apgar score >6 OR (95% CI)	Hypoxic-ischaemic Encephalopathy OR (95% CI)
rs2284411	0.92 (0.80 to 1.05)	0.92 (0.82 to 1.04)	1.02 (0.60 to 1.76)
rs2498804	1.08 (0.94 to 1.23)	0.98 (0.87 to 1.11)	1.13 (0.65 to 1.95)
rs1835740	1.17 (1.01 to 1.35)	1.17 (1.03 to 1.34)	0.92 (0.49 to 1.75)

Values are OR (95% CI)

Table 5. Logistic regression models for the association between the three candidate SNPs and the need for resuscitation, for each increasing allele.

SNP	Unadjusted OR (95% CI)	Adjusted for antenatal* factors OR (95% CI)	Adjusted for antenatal* and Intrapartum** factors OR (95% CI)
rs2284411	0.92 (0.80 to 1.05)	0.91 (0.79 to 1.04)	0.91 (0.79 to 1.06)
rs2498804	1.08 (0.94 to 1.23)	1.09 (0.95 to 1.25)	1.09 (0.94 to 1.26)
rs1835740	1.17 (1.01 to 1.35)	1.17 (1.01 to 1.36)	1.18 (1.00 to 1.38)

* Adjusted for gender, parity and maternal hypertension

** Adjusted for gestational age, birth weight, length and head circumference, mode of birth and neonatal sepsis

Values are OR (95% CI)